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Uncoupling Stress Granule Assembly and Translation Initiation Inhibition

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Cytoplasmic stress granules (SGs) are specialized regulatory sites of mRNA translation that form under different stress conditions known to inhibit translation initiation. The formation of SG occurs via two pathways; the eukaryotic initiation factor (eIF) 2α phosphorylation-dependent pathway mediated by stress and the eIF2α phosphorylation-independent pathway mediated by inactivation of the translation initiation factors eIF4A and eIF4G. In this study, we investigated the effects of targeting different translation initiation factors and steps in SG formation in HeLa cells. By depleting eIF2α, we demonstrate that reduced levels of the eIF2.GTP.Met-tRNAi^{Met} ternary translation initiation complexes is sufficient to induce SGs. Likewise, reduced levels of eIF4B, eIF4H, or polyA-binding protein, also trigger SG formation. In contrast, depletion of the cap-binding protein eIF4E or preventing its assembly into eIF4F results in modest SG formation. Intriguingly, interfering with the last step of translation initiation by blocking the recruitment of 60S ribosome either with 2-(4-methyl-2,6-dinitroanilino)-*N*-methylpropionamideis or through depletion of the large ribosomal subunits protein L28 does not induce SG assembly. Our study identifies translation initiation steps and factors involved in SG formation as well as those that can be targeted without induction of SGs.

INTRODUCTION

Tight regulation of translation initiation is critical for proper control of cell response to environmental and pathological stresses. Initiation of translation involves the recognition of mRNAs by the translation-initiation factors (eukaryotic initiation factors [eIFs]) and formation of 80S ribosome on the mRNA (Kapp and Lorsch, 2004; Marintchev and Wagner, 2004). The earliest step of initiation involves the formation of eIF2.GTP.Met-tRNAi^{Met} ternary complex (TC) and its association with the 40S ribosome resulting in 43S preinitiation complex formation. In the second step (ribosomal recruitment step), the 43S is recruited to mRNA by the eIF4F complex. This leads to the formation of the 48S initiation complex. Once the 40S ribosome is positioned at the initiation codon, the 60S ribosome is recruited to form an 80S complex competent for translation. eIF4F consists of the cap-binding protein eIF4E, the scaffolding protein eIF4G, and the RNA helicase eIF4A known to unwind secondary structures in the 5′ untranslated region of mRNAs (Gingras *et al*., 1999; Sonenberg and Dever, 2003). In addition to eIF4F, other factors are involved in the stabilization of ribosome–mRNA interactions. Whereas the polyA-binding protein (PABP) promotes this stabilization through its interaction with eIF4G, eIF4B and eIF4H are RNA chaperones that assist the unwinding activity of eIF4A (Kahvejian *et al*., 2001; Sonenberg and Dever, 2003; Svitkin and Sonenberg, 2006).

Translation initiation is regulated by the availability of the eIF2.GTP.Met-tRNAi^{Met} TC and the eIF4F complex (Gebauer and Hentze, 2004). The rate-limiting step for the assembly of the TC is the binding of guanosine triphosphate (GTP) to eIF2α. Phosphorylation of the α-subunit of eIF2 at residue Ser51 blocks the exchange of GDP for GTP during recycling of the eIF2 complex by eIF2B, preventing the formation of a functional ternary complex and leading to reduced translation of most mRNAs (Gebauer and Hentze, 2004). This modification is a well characterized mechanism of translation initiation inhibition that occurs upon induction of cellular stress (Wek *et al*., 2006).

Stress response involves a reprogramming of gene expression that is essential for cell survival. Central to this response is the formation of stress granules (SGs). SGs are cytoplasmic bodies that regulate mRNA translation and turnover upon stress (Anderson and Kedersha, 2008). They are induced in response to a variety of stresses known to inhibit translation initiation. These include oxidative stress, heat shock, viral infection, and proteosome inhibition (Kedersha *et al*., 1999; McInerney *et al*., 2005; Mazroui *et al*., 2007). On induction of stress, mRNA and associated proteins (messenger ribonucleoprotein particles [mRNPs]) are rapidly recruited from translating ribosomes into SGs as untranslated mRNP. Once the stress is over, SGs gradually dis-

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assemble leading to the recovery of translation (Anderson and Kedersha, 2008).

In addition to mRNAs, SGs contain the small but not the large ribosomal subunits, translation initiation factors and various RNA-binding proteins. The RNA binding components of SGs include proteins involved in 1) translation repression, such as TIA/TIAR and fragile X mental retardation protein (FMRP) (Kedersha *et al*., 1999; Mazroui *et al*., 2002); 2) mRNA stabilization, such as HuR (Gallouzi *et al*., 2000); and 3) mRNA decay, such as TTP and BRF1 (Kedersha *et al*., 2002, 2005). Interestingly, many of these SG-associated proteins are also known to be components of P-bodies, sites where mRNA decay is believed to occur (Kedersha *et al*., 2005). This raises the possibility that a cross talk between SGs and P-bodies is required to ensure cell protection against stress. The exclusion of the large ribosomal subunits as well as elongation factors from SGs and P-bodies is intriguing but might reflect an antagonistic role of these factors in their assembly.

The mechanism of P-body formation is largely unknown, although their number and size increase when the 5′-to-3′ mRNA decay is blocked or when translation initiation is inhibited by stress (Kedersha *et al*., 2005; Anderson and Kedersha, 2006). The formation of P-bodies can also be blocked by treatment with translation elongation inhibitors known to trap mRNPs with polysomes (Brengues *et al*., 2005; Teixeira *et al*., 2005). These inhibitors also block SG formation, indicating that the formation of P-bodies and SGs share some common pathways.

Until recently, it was thought that the formation of SGs can be initiated only via a mechanism that requires the phosphorylation of the α-subunit of the translation initiation factor eIF2 at residue Ser51 and which subsequently reduces the levels of eIF2.GTP.Met-tRNAi^{Met} TC (Anderson and Kedersha, 2006). Whether the inhibition of TC formation itself triggers the formation of SGs or whether it requires the phosphorylation of eIF2α is currently unknown. We and others have recently described a novel pathway of SG assembly that is independent of eIF2α modification (Dang *et al*., 2006; Mazroui *et al*., 2006). In these studies, SGs were induced upon depletion of eIF4A or its inactivation by two novel small molecules inhibitors of translation initiation, pateamine and hippuristanol. We have also reported that poliovirus-mediated cleavage of eIF4G triggers SG formation (Mazroui *et al*., 2006). These studies indicate that inhibition of translation initiation at the level of the small ribosome recruitment step through inactivation of eIF4A or eIF4G is sufficient to induce the formation of SGs. However, the exact translation initiation steps and factors involved in SG assembly are still elusive. In this study, we delineated the steps of translation initiation involved in SG formation. Our results revealed that SG formation can be uncoupled from inhibition of translation initiation.

MATERIALS AND METHODS

Cell Lines and Cultures

HeLa and lung Calu-1 cancer cells were obtained from American Type Culture Collection (Manassas, VA). Wild-type (WT) mouse embryonic fibroblasts (MEFs), and MEFs harboring the mutation eIF2$\alpha^{S51A/S51A}$ were described previously (Scheuner *et al*., 2001). Cells were cultured in DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), penicillin, and streptomycin (Sigma-Aldrich).

Drugs and Drug Treatments

NSC 119893 and 2-(4-methyl-2,6-dinitroanilino)-*N*-methylpropionamideis (MDMP) were dissolved in dimethyl sulfoxide (DMSO) at 20 and 10 mM stock solutions, respectively, and stored at −20°C. 4EGI-1 was purchased from Alexis Biochemicals (San Diego, CA), dissolved in DMSO at 4 mM stock solution, and stored at −20°C. Arsenite and cycloheximide were obtained from Sigma-Aldrich. All drug treatments were performed when cells reached 60–80% confluence.

[^{35}S]Methionine Labeling

Cells in a six-well plate were labeled 30 min with 1 ml of methionine-free DMEM (Sigma-Aldrich) supplemented with 10% FBS and with 50 μCi/ml [^{35}S]methionine (Easy Tag; PerkinElmer Life and Analytical Sciences, Boston, MA).

Antibodies

Phospho-specific anti-eIF2α and the pan anti-eIF2 were purchased from Cell Signaling Technology (Danvers, MA). Anti-HuR, anti-G3BP, anti-FMRP, anti-FXR1, anti-Dcp1a, anti-XRN1, anti-eIF4A have been described previously (Mazroui *et al*., 2006, 2007). Anti-RCK and anti-L28 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-eIF4E was obtained from BD Biosciences Transduction Laboratories (Lexington, KY). Anti-PABP, anti-eIF4B, and anti-eIF4H antibodies were obtained from Cell Signaling Technology.

Small Interfering RNA (siRNA) Transfections

All siRNA were purchased as validated oligonucleotides (oligos) from QIAGEN (Valencia, CA). eIF4E-siRNAs were obtained from Dharmacon RNA Technologies (Lafayette, CO). siRNA transfections were performed in HeLa cells essentially as documented previously (Mazroui *et al*., 2008). siRNA transfections were performed using Hiperfect reagent following the manufacturer's protocol (QIAGEN and Dharmacon RNA Technologies). Twenty-four hours before transfections, cells were trypsinized and plated to obtain 60–80% confluence the day after. For a six-well plate, annealed duplex was used at a final concentration of 20 nM. Forty eight hours after transfection, cells were either fixed and processed for immunofluorescence or harvested for protein extraction. The efficiency of the knockdown was determined by quantification of the signal on films using ImageQuant (GE Healthcare, Chalfont St. Giles, Buckinghamshire, United Kingdom).

Fluorescence Microscopy

Immunofluorescence experiments were performed following the previously described protocol (Mazroui *et al*., 2002). Essentially, after fixation and permeabilization, cells were incubated with primary antibodies diluted in 0.1% Tween 20/1× phosphate-buffered saline for 1 h at room temperature. After washing, cells were incubated with goat anti-mouse/rabbit immunoglobulin G (H + L) secondary antibodies coupled to Alexa Fluor 488/594. Fluorescence was visualized using an Axiovision microscope (Olympus, Tokyo, Japan) equipped with AxioCam HR digital camera. Images were compiled using Adobe Photoshop software (Adobe Systems, Mountain View, CA).

Polysomal Profiles Analyses

Polysomal profiles were performed as follow: HeLa cells were grown in 100-mm tissue culture dishes to 80% confluence, harvested, and resuspended in 1 ml of polysomal buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1.25 mM MgCl, 5 U/ml RNAsine [GE Healthcare], EDTA-free protease inhibitor cocktail [Complete; Roche, Indianapolis, IN], and 1 mM dithiothreitol), and Nonidet P-40 was added to a final concentration of 1% for lysis of 15 min on ice. Extracts were clarified by centrifugation at 12,000 × *g* for 20 min at 4°C. Cytoplasmic extracts were loaded on each 10–60% linear sucrose gradient and further analyzed as described previously (Mazroui *et al*., 2002, 2003).

In Vitro Translations

In vitro transcription and translations of bicistronic Ren/cricket paralysis virus (CrPV)/firefly luciferase (FF) mRNA were described previously (Robert *et al*., 2006). Translation was performed in presence of [^{35}S]methionine and translated proteins were separated on 10% polyacrylamide/SDS gels. Proteins were detected by autoradiography of the gels exposed to X-Omat films (Fastman; Eastman Kodak, Rochester, NY).

Ribosome Binding Experiments

These assays were done following previously published protocols (Robert *et al*., 2006). Briefly, ^{32}P-labeled CrPV internal ribosome entry sequence (IRES)-containing transcript was incubated in Krebs-2 extracts at 30°C for 10 min in the presence of cycloheximide and in presence or absence of NSC 119893, D-MDMP or L-MDMP. Initiation complexes formed on mRNAs were resolved on 5–20% sucrose gradients by centrifugation at 37,000 rpm/4 h in an SW 40 rotor.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

RT-PCR reactions were performed using the Quantitect Reverse transcriptase (QIAGEN). Each reaction contain 2 μl of RNA at 500 ng/μl, 10 μl of RNase-free water, 2 μl of genomic DNA Wipeout buffer 7×, 4 μl of Quantiscript RT buffer 5×, 1 μl of RT Primer Mix, and 1 μl of Quantiscript reverse transcriptase.

Real-time PCR reactions were prepared using the Power SYBR Green PCR Master mix (Applied Biosystems, Foster City, CA) in a total volume of 25 μl: 12.5 μl of PCR Master Mix, 0.67 μl of forward primer at 3.75 μM, 0.67 μl of reverse primer at 3.75 μM, 9.2 μl of Milli-Q water (Millipore, Billerica, MA), and 2 μl of RT-PCR. Reactions were run and data analyzed on the MX3000 qRT-PCR system (Applied Biosystems), with a four-stage program: first stage (2-min incubation at

50°C), second stage (10-min incubation at 95°C), followed by a two-step reaction in the third stage (95°C × 15 s and 55°C × 60 s for 40 cycles), and a fourth stage of three-step reaction (95°C × 15 s, 60°C × 20 s and 95°C × 15 s).

For preparing templates corresponding to the bcl2 mRNA, the oligonucleotide pairs used were 5′-GCCCTGTGGATGACTGAGTA-3′ (forward primer) and 5′-GAGACAGCCAGGAGAAATCA-3′(reverse primer). For preparing templates corresponding to the caspase-9 mRNA, the oligonucleotide pairs used were 5′-TCCTGCTTAGAGGACACAGG-3′ (forward primer) and 5′-CAAATCCTCCAGAACCAATG-3′ (reverse primer). For preparing templates corresponding to the hsp70 mRNA, the oligonucleotide pairs used were 5′-AAGAGCATCAACCCCGACG-3′ (forward primer) and 5′-TCTCCAGCCCCAGCGACAG-3 (reverse primer).

RESULTS

Formation of SGs Can Be Induced by Inhibiting the Formation of the eIF2α.GTP.Met-tRNA$_i^{Met}$ Ternary Complex

Under some stress conditions such as oxidative stress, the formation of SGs is triggered by eIF2α phosphorylation (Kedersha and Anderson, 2007). This modification converts eIF2 from a substrate to a competitive inhibitor of eIF2B, preventing guanine nucleotide exchange, and inhibiting TC formation. To demonstrate that inhibition of TC, and not the phosphorylation of eIF2α per se, is responsible for triggering SGs, we tested the effect of NSC 119893, a compound that impairs TC (formation (Robert *et al*., 2006). NSC 119893 specifically prevents the association of Met-tRNA$_i^{Met}$ with eIF2α and thus limits the availability of TC (Robert *et al*., 2006). Treatment of HeLa cells with NSC 119893 efficiently induced SG formation (90%) as assessed by immunofluorescence using the SG markers HuR, G3BP, FMRP, and FXR1 (Figure 1A). The same results were obtained using other human transformed cells (data not shown). Previous studies showed that stress such as arsenite treatment can increase the numbers of P-bodies, whereas proteasome inhibition temporarily alters their formation (Kedersha *et al*.,

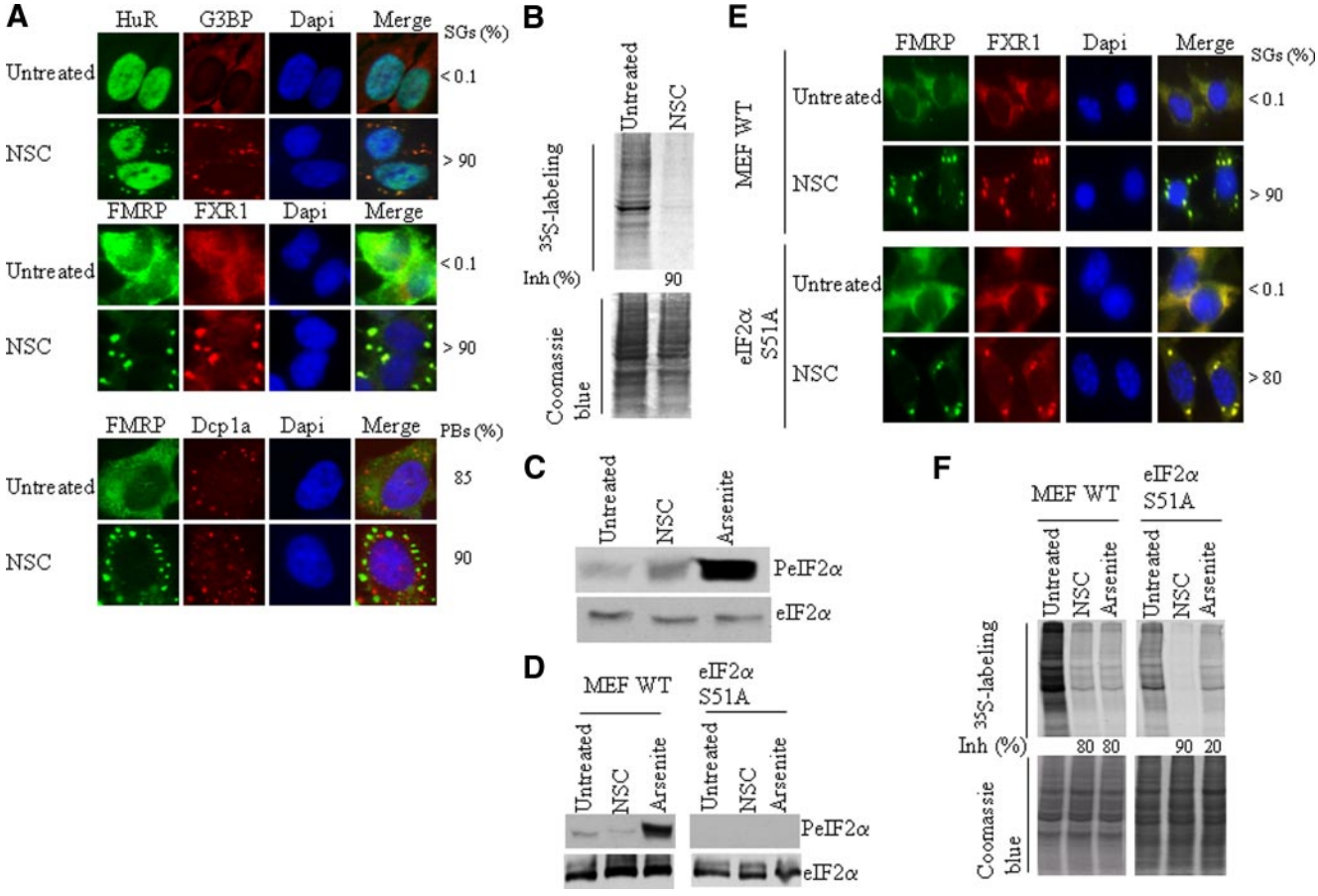

Figure 1. Inhibition of TC formation via NSC 119893 induces SG formation and occurs independently of eIF2 αphosphorylation. (A) HeLa cells were treated with 10 μM NSC 119893 for 60 min, fixed, permeabilized, and processed for immunofluorescence by using antibodies against different SG markers. Anti-Dcp1a antibodies were used to detect P-bodies. 4,6-Diamidino-2-phenylindole (Dapi) is used as nuclei marker. The percentage of cells harboring SGs (≥5 granules/cell), or P-bodies (PBs) (≥4 bodies/cell) is indicated to the right of the figure and is representative of the analysis of five different fields in three independent experiments for a total of 1000 cells counted. Pictures in the top and middle panels were taken using 40× objective. P-bodies pictures (bottom) were taken using a 60× oil immersion objective. (B) HeLa cells were treated with 10 μM NSC 119893 for 30 min and then incubated with [^{35}S]methionine (50 μCi/ml) for another 30 min. Proteins were resolved by SDS-polyacrylamide gel, stained with Coomassie Blue (bottom), and detected by autoradiography (top). (C) HeLa cells were treated with 10 μM NSC 119893 or with 0.5 mM arsenite for 30 min, and the level of phospho-eIF2α was analyzed by Western blotting using antibodies specific to the phosphorylated form. Detection of the total levels of eIF2α is shown in the bottom panel and serves as a loading control. (D) WT and eIF2α$^{S51A/S51A}$ MEFs were treated with 10 μM NSC 119893 or with 0.5 mM arsenite for 30 min, and proteins were analyzed for the phosphorylation of eIF2α by Western blotting as described in C. (E) WT and eIF2α$^{S51A/S51A}$ MEFs were treated with 10 μM NSC 119893 for 60 min, and SGs were visualized as described above using anti-HuR and anti-FXR1 antibodies. The percentage of SGs formed in each cell line is indicated. (F) WT and eIF2α$^{S51A/S51A}$ MEFs were incubated with 10 μM NSC 119893 or with 0.5 mM arsenite for 30 min then with [^{35}S]methionine (50 μCi/ml) for an additional 30 min. Proteins were prepared and analyzed as described in B.

2005; Mazroui *et al*., 2007). Our results show that NSC 119893 does not affect the proportion of cells harboring P-bodies as assessed by immunofluorescence using different P-body markers (Figure 1A, bottom, and Supplemental Figure 1). Consistent with previous data (Robert *et al*., 2006), NSC 119893 inhibits global translation in HeLa cells as assessed by metabolic labeling (Figure 1B). Western blot analysis of protein extracts prepared from NSC 119893-treated HeLa cells detected only trace amounts of phospho-eIF2α (Figure 1C). The same results were obtained using WT MEFs (Figure 1, D–F), suggesting that NSC 119893 induces the formation of SGs independently of eIF2α phosphorylation. To confirm this, we used eIF2α^{S51A} MEFs in which eIF2α Ser 51 has been mutated to Ala and which cannot be phosphorylated (Scheuner *et al*., 2001). In these cells, NSC 119893 reduces general translation and induces the SG formation as efficiently as in WT MEF (Figure 1, E and F). Overall, our results show that targeting TC formation is sufficient to induce SG assembly.

Because NSC 119893 targets TC formation by preventing eIF2α and Met-tRNA$_i^{Met}$ association, we tested whether depleting eIF2α would mimic the effect of NSC 119893 in SG formation. HeLa cells were treated with a siRNA directed to eIF2α (eIF2α-1) or with a control siRNA. Transfection of HeLa cells with eIF2α-1, but not with the control siRNA, induced SGs in ~6–8% of total cells as determined by immunofluorescence using the SG markers FMRP and FXR1 (Figure 2A, top). Similar results were obtained using a second eIF2α-siRNA that targets a different region of eIF2α mRNA (data not shown). Moreover, SGs were evident in cells where eIF2α was efficiently depleted as assessed by immunofluorescence using anti-eIF2α and anti-FMRP antibodies (Figure 2A, middle). Consistent with NSC data, depletion of eIF2α under our experimental conditions has no effect on P-bodies (Figure 2A, bottom). Western blot analysis of the eIF2α protein indicated that we achieved ~50% depletion of eIF2α (Figure 2B) and metabolic labeling indicated that general translation was reduced by ~45% in eIF2α-1–treated cells (Figure 2C). Hence, interfering with the TC formation by either depleting eIF2α or preventing its association with Met-tRNA$_i^{Met}$ induces SG formation.

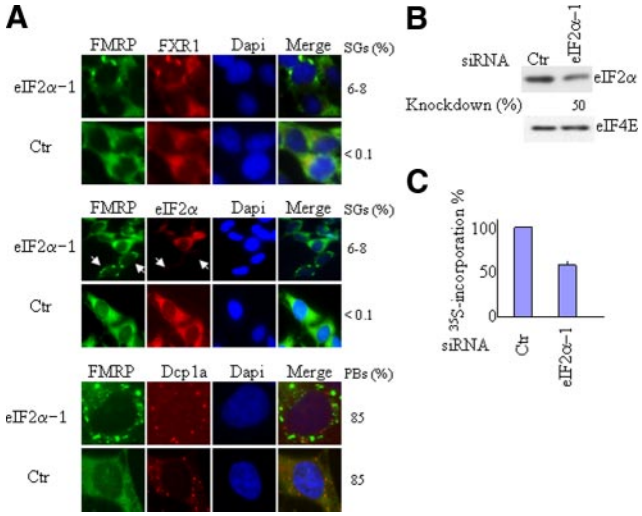

Figure 2. Reducing eIF2α levels by siRNA induces SG formation. (A) Forty-eight hours after transfection with eIF2α-1 or control siRNA, HeLa cells were processed for immunofluorescence by using antibodies against FMRP, FXR1, eIF2α, and Dcp1a. The percentage of cells forming SGs (≥5 granules/cell) or P-bodies (≥4 bodies/cell) is indicated on the right and is representative of the analysis of five different fields in three independent experiments for a total of 1000 cells counted. eIF2α-depleted cells are indicated by arrows. Pictures in the top and middle panels were taken using 40× objective. P-bodies pictures (bottom) were taken using a 60× oil immersion objective. (B) Western blot analysis of proteins extracted from cells treated with eIF2α-1 or control siRNA. The membrane was incubated with anti-eIF2α (top) and as a control with eIF4E (bottom) antibodies. The percentage of eIF2α knockdown was determined by quantitation of the signal on films by using ImageQuant (GE Healthcare). (C) Knockdown of eIF2α inhibits translation in vivo. Forty-eight hours after treatment with eIF2α or control siRNA, cells were incubated 30 min with 50 μCi/ml [^{35}S]methionine. The percentage of translation inhibition was measured by quantification of trichloroacetic acid-precipitable counts and represents the average of three independent experiments.

Interfering with eIF4B, eIF4H, or PABP, but Not with eIF4E, Triggers SG Assembly

We have recently reported that altering the activity of two components of the eIF4F complex, eIF4A or eIF4G, induces SG formation, and this occurs independently of eIF2α phosphorylation (Mazroui *et al*., 2006). This suggested that interfering with the ribosome–mRNA association is sufficient to trigger SG formation. In addition to eIF4A and eIF4G, the ribosome-mRNA association step involves the activity of eIF4B, eIF4H, PABP, and eIF4E. We assessed the effects of depleting these factors on SG assembly. HeLa cells were treated with specific siRNAs directed to eIF4B (eIF4B-1), eIF4H (eIF4H-1), PABP (PABP-1), eIF4E (eIF4E-1), or with a control siRNA, and SG formation was assessed by immunofluorescence using specific SG markers. As shown in Figure 3A, treatment of cells with either eIF4B-1, eIF4H-1, or PABP-1 resulted in induction of SGs (6–8% of treated cells formed visible SGs). Depletion of eIF4E or treatment of cells with the control siRNA was less efficient in SG induction (<0.1% of treated cells form SGs). Depletion of eIF4E and PABP was evident by immunofluorescence using anti-eIF4E and PABP antibodies, confirming that depletion of eIF4E does not induce SGs, whereas PABP-depleted cells does (Figure 3B). Despite our efforts, we could not assess depletion of eIF4B and eIF4H by immunofluorescence. However, our Western blot analysis show that we achieved depletion of ~50% of eIF4B, 85% of eIF4H, 70% of PABP, and 80% of eIF4E, after treatment with their respective siRNAs (Figure 3C). Depletion of either factor did not, however, affect the total amounts of SG markers such as FMRP and G3BP (Figure 3D). Consistent with the role of eIF4B, eIF4H, PABP, and eIF4E in translation, reduced levels of any of these factors inhibited translation by 40–70% as assessed by metabolic labeling (Figure 3E). Previous studies showed that depletion of eIF4E by siRNA or short hairpin RNA (shRNA) results in only a modest inhibition of global translation (Svitkin *et al*., 2005; Lin *et al*., 2008). This discrepancy may be attributed to the cell types used. Nonetheless, our results show that targeting the ribosome recruitment step by inactivation of eIF4B, eIF4H, or PABP induces SG formation, whereas depletion of eIF4E is not efficient in SG induction. We sought to confirm those results by another approach. A recent study identified the compound 4EGI-1 in a high-throughput screen that specifically binds eIF4E and disrupts its interaction with eIF4G, which in turn reduces cap-dependent translation of reporter mRNAs (Moerke *et al*., 2007). However, the effect of 4EGI-1 on global translation initiation was not tested in that study. Treatment of HeLa cells with 4EGI-1 inhibits translation by >80% as assessed by metabolic labeling (Figure 4A) and reduces the peak of polysomes as assessed by sucrose gradient fractionation analysis. This re-

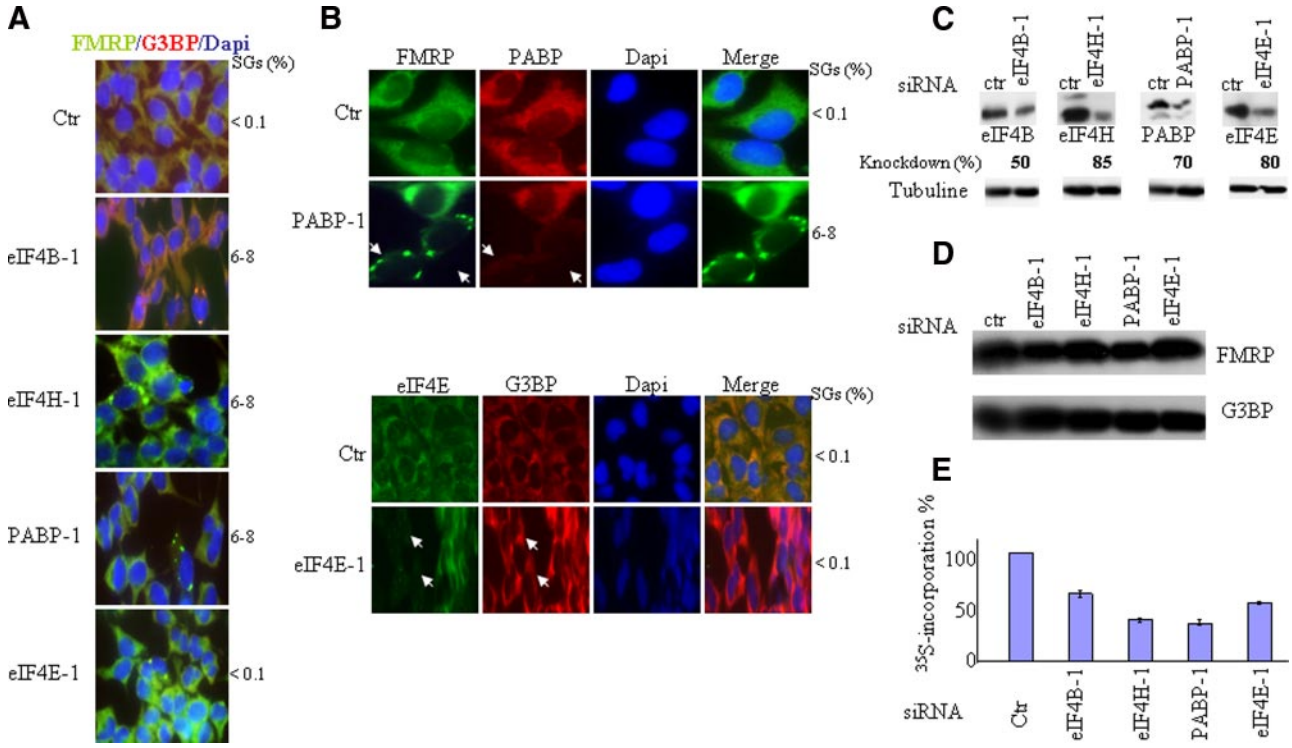

Figure 3. Depletion of eIF4B, eIF4H, or PABP induces SG formation, whereas depletion of eIF4E has a minimal effect on SG formation. (A) HeLa cells were treated with eIF4B-1, eIF4H-1, PABP-1, eIF4E-1, or with a control siRNA, and then processed for immunodetection of SGs by using anti-FMRP and anti-G3BP antibodies. The percentage of cells forming SGs (≥5 granules/cell) is indicated and is representative of three independent experiments for a total of 1000 cells counted. (B) HeLa cells were treated with siRNA PABP-1, eIF4E-1, or with a control siRNA. PABP-depleted cells (indicated by arrows) were visualized by immunofluorescence using anti-PABP antibodies, and the formation of SGs in these cells was detected with anti-FMRP antibodies. eIF4E-depleted cells (indicated with arrows) were detected using anti-eIF4E antibodies, and the absence of SGs in these cells was confirmed with anti-G3BP antibodies. (C and D) Western blot analysis of proteins extracted from cells treated with eIF4B-1, eIF4H-1, PABP-1, eIF4E-1, or siRNA control. (C) The membrane was incubated with the indicated antibodies and as a control with anti-tubulin (bottom) antibodies. (D) The membrane was incubated with anti-FMRP (top) and anti-G3BP (bottom) antibodies. (E) Depletion of eIF4B, eIF4H, PABP, or eIF4E reduces translation in vivo. After treatment with eIF4B-1, eIF4H-1, PABP-1, eIF4E-1, or control siRNA, cells were incubated 30 min with 50 μCi/ml [^{35}S]methionine. The percentage of translation inhibition was measured by quantification of trichloroacetic acid-precipitable counts and represents the average of three independent experiments.

duction was accompanied by an accumulation of the 80S peak consistent with translation inhibition at the initiation level (Figure 4B). This result is not seen by reducing of eIF4E levels by shRNA (Lin *et al.*, 2008) and may indicate additional off-target effects of 4EGI-1 (Moerke *et al.*, 2007). Nonetheless, 4EGI-1 treatment only induced SG formation in 2–5% of treated HeLa cells (Figure 4C, top and middle); yet, it does not affect the expression of SG markers such FMRP and G3BP (Figure 4D). We concluded that inactivation of the cap-binding protein eIF4E is at best a weak inducer of SG assembly. Moreover, 4EGI-1 treatment does not affect the proportion of HeLa cells displaying P-bodies (Figure 4C, bottom, and Supplemental Figure 1), which is consistent with previous studies showing that 4EGI-1 does not alter the stability of different mRNAs (Moerke *et al.*, 2007). Our study also suggests that the formation of P-bodies is not linked to the activity of eIF4E.

Formation of SGs Occurs Independently of the 60S Ribosomal Joining Step of Translation Initiation

The last step of translation initiation involves the recruitment of the 60S to the 40S, forming an 80S complex competent for translation. Whether inhibition of this step results in SG formation has not been investigated. To address this question, we first assessed the formation of SGs upon depletion of the large ribosomal protein L28 or the translation initiation factor eIF5B, which is known to promote the 60S joining step. After treatment with siRNAs specific to either L28 (L28-1) or to eIF5B (eIF5B-1), the formation of SGs was assessed by immunofluorescence. The results show that <0.1% of cells treated with either siRNA formed SGs (Figure 5A). Although depletion of L28 was not evident by immunofluorescence, our Western blots analysis shows that L28 protein was depleted by 80% (Figure 5B). Due to the absence of suitable anti-eIF5B antibodies, we assessed the effect of eIF5B-1 siRNA on the target mRNA by qRT-PCR analysis. The results show that eIF5B-1 efficiently targeted eIF5B mRNA degradation (Figure 5C). Metabolic labeling revealed that depletion of L28 and eIF5B decreased general translation by 65 and 45%, respectively (Figure 5D). This result indicates that L28 and eIF5B proteins play an important role in translation yet their depletion does not trigger SG assembly. This suggests that the assembly of SGs may occur independently of the 60S machinery.

We further investigated the relationship between the formation of SGs and the 60S recruitment step using a pharmacological approach. To our knowledge, the only drug that can specifically block the binding of 60S with 40S ribosomes is MDMP (Baxter *et al.*, 1973). The L-isomer of MDMP was used as a negative control in our experiments. We assessed

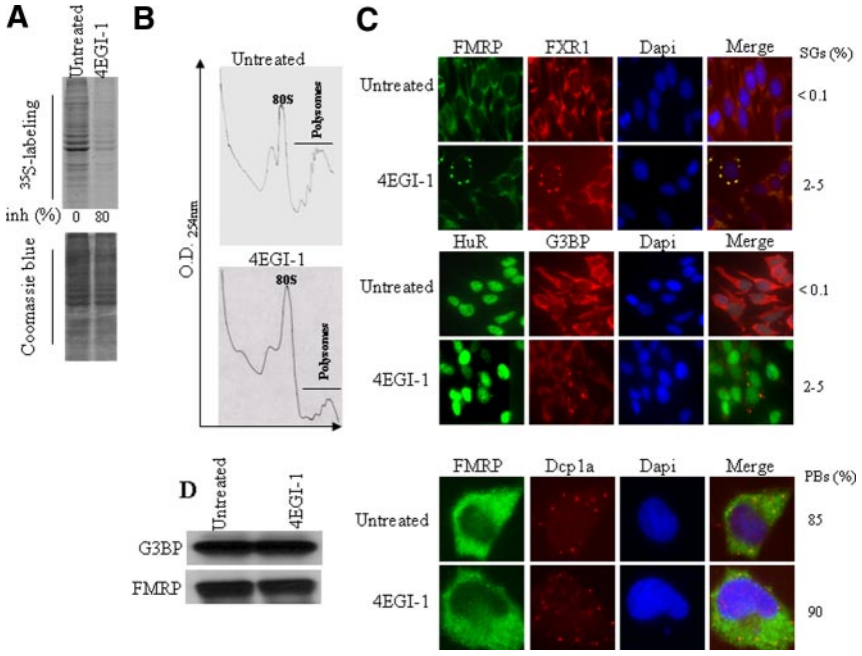

Figure 4. Chemical targeting of eIF4E weakly induces SG formation. (A) 4EGI-1 treatment inhibits cellular translation. HeLa cells were treated with 100 μM 4EGI-1 for 5 h and 30 min and then labeled with [^{35}S] methionine (50 μCi/ml) for another 30 min. Proteins were resolved by SDS-polyacrylamide gel, stained with Coomassie Blue (bottom), and detected by autoradiography (top). (B) 4EGI-1 induces polysome disassembly. Cytoplasmic extracts of untreated (top) or 4EGI-1-treated HeLa cells (bottom) were prepared and fractionated on 10–60% sucrose gradients. The polysome profile was monitored by measuring the OD_{254}. (C) HeLa cells were incubated with 100 μM 4EGI-1 for 6 h, fixed, permeabilized, and processed for immunofluorescence by using anti-SG marker antibodies as described above. Anti-Dcp1a antibodies were used to detect P-bodies. The percentage of cells harboring SGs (≥5 granules/cell), or P-bodies (PBs) (≥4 bodies/cell) is indicated to the right of the figure and is representative of the analysis of five different fields in three independent experiments for a total of 1000 cells counted. Pictures in the top and middle panels were taken using 40× objective. P-bodies pictures (bottom) were taken using a 60× oil immersion objective. (D) After a 6-h treatment with 100 μM 4EGI-1, cells were collected and proteins content was analyzed by Western blot for the expression of FMRP and G3BP by using the corresponding antibodies.

the effect of MDMP on the assembly of the 80S complex on CrPV IRES by using Krebs-2 extracts. The CrPV IRES does not require any of the canonical translation initiation factors for 80S complex formation (Pisarev *et al*., 2005; Robert *et al*., 2006; Cencic *et al*., 2007). This features of the CrPV IRES prompted us to assess the consequence of MDMP on 80S assembly. Cycloheximide (CHX) was used to trap 80S complexes. As shown in Figure 6A, MDMP (MDMP + CHX)

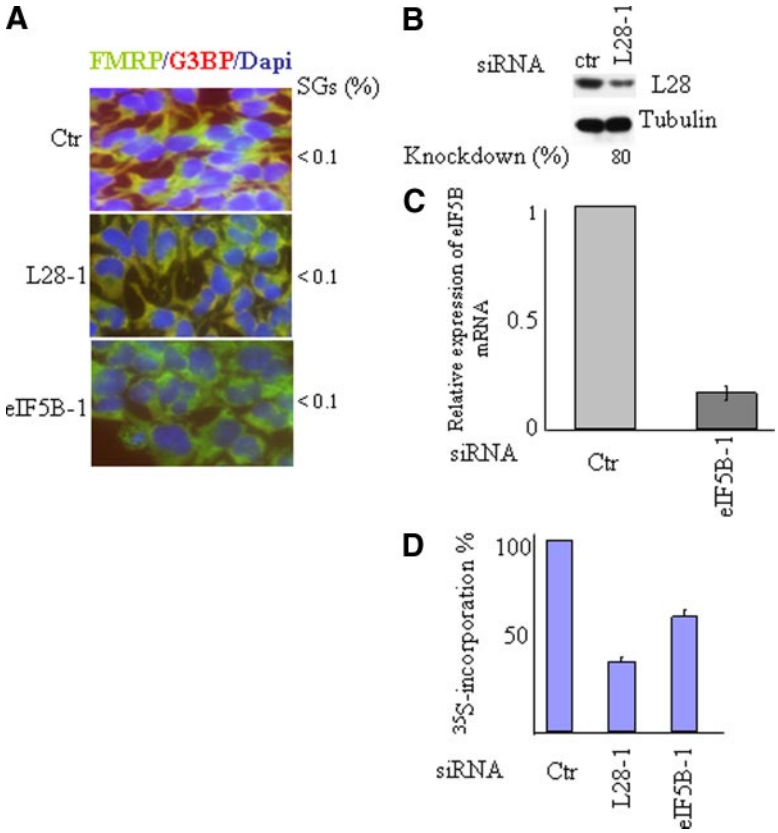

Figure 5. Depletion of L28 or eIF5B does not induce SG formation. (A) HeLa cells were treated with L28-1, eIF5B-1, or with a control siRNA, and then processed for immunodetection of SGs. (B) Western blot analysis of proteins extracted from cells treated with L28-1 or control siRNA. The membrane was incubated with the indicated antibodies (top) and as a control with anti-tubulin (bottom) antibodies. (C) qRT-PCR of eIF5B mRNA. After 48-h treatment of HeLa cells with eIF5B-1 or with a control siRNA, isolation of mRNAs, and preparation of cDNA, the amounts of eIF5B mRNAs relative to 18S rRNA was quantified by real time-PCR using the ΔΔCt method. The results are mean of triplicate measurements, with error bars corresponding to SEM. (D) Depletion of L28 or eIF5B reduces translation in vivo. After treatment with L28-1, eIF5B-1, or control siRNA, cells were incubated 30 min with 50 μCi/ml [^{35}S]methionine. The percentage of translation inhibition was measured by quantification of trichloroacetic acid-precipitable counts and represents the average of three independent experiments for a total of 1000 cells counted.

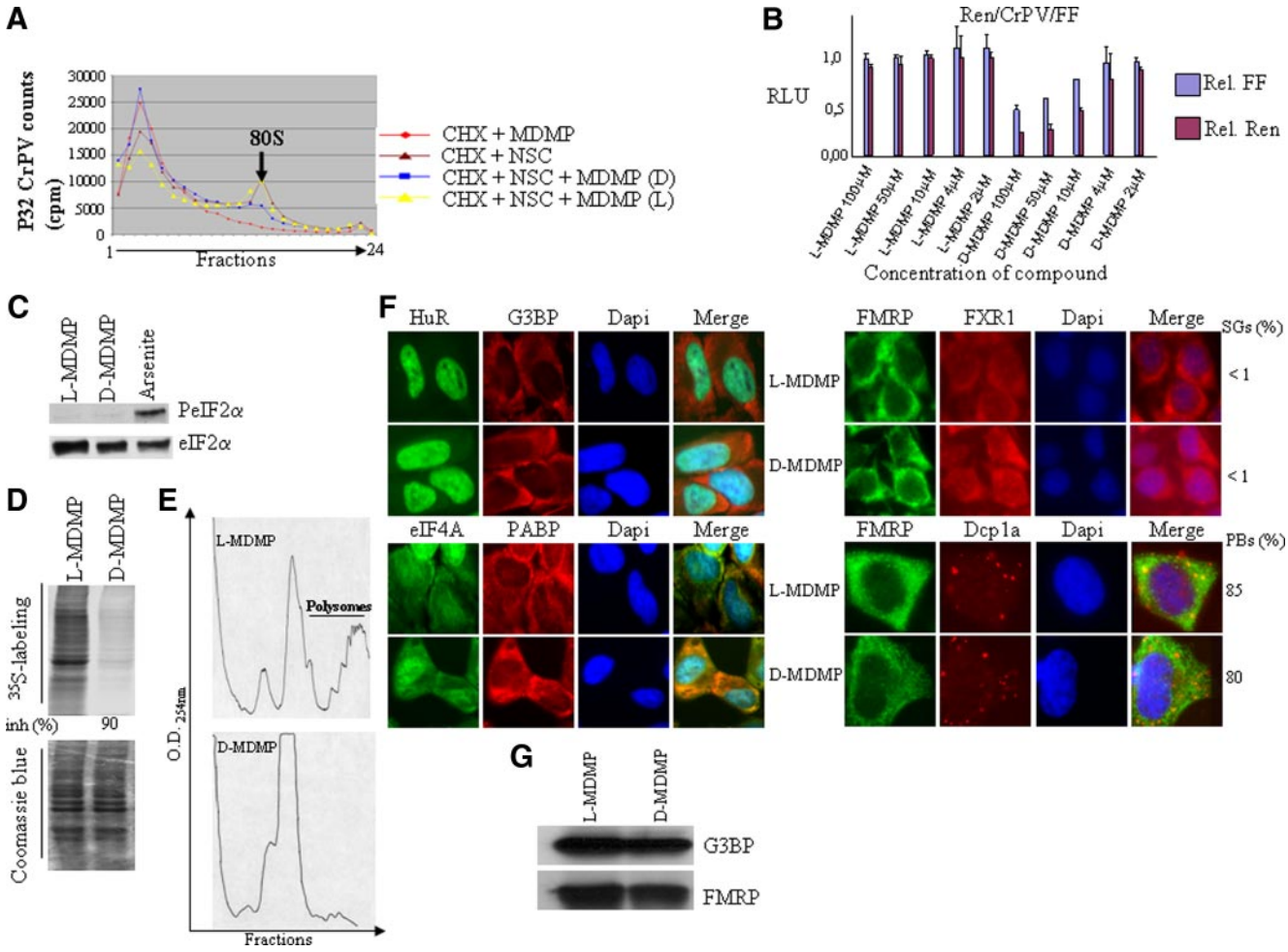

Figure 6. SG formation occurs independently of the 60S recruitment step of translation initiation. (A) MDMP prevents the 80S formation. Krebs-2 extracts were preincubated with 100 μM MDMP and 0.6 mM cycloheximide (red line); with 50 μM NSC 119893 and 0.6 mM cycloheximide (brown line); with 100 μM MDMP, 0.6 mM cycloheximide, and 50 μM NSC 119893 (blue line); or with 100 μM inactive L-MDMP, 0.6 mM cycloheximide, and 50 μM NSC 119893 (yellow line) at 30°C for 5 min. Reactions were then mixed with ^{32}P-radiolabeled CrPV IRES RNA and incubated for 10 min at 30°C. Complexes were sedimented by centrifugation through sucrose gradient. (B) MDMP inhibits cap-dependent and CrPV-mediated translation. Translations were performed with 10 μg/ml bicistronic Ren/CrPV/FF mRNA in the presence of [^{35}S]methionine, D-MDMP, or L-MDMP at the indicated concentrations. Luciferase activity was measured with a luminometer. Error bars correspond to SEM of three independent experiments. (C) Cells were treated with 100 μM either D-MDMP or L-MDMP for 2 h or with 0.5 mM arsenite for 30 min, and proteins were analyzed by Western blotting using anti-phosho-eIF2α (top) or anti-eIF2α (bottom) antibodies (D) HeLa cells were treated with either 100 μM D-MDMP or L-MDMP or with 0.5 mM arsenite for 1 h and then incubated with [^{35}S]methionine (50 μCi/ml) for another 60 min. (E) MDMP induces polysome disassembly. Cytoplasmic extracts of HeLa cells treated with 100 μM D-MDMP (bottom) or with 100 μM L-MDMP (top) were prepared and fractionated on 10–60% sucrose gradients. The polysome profile was monitored by measuring the OD_{254}. (F) MDMP does not induce SGs. HeLa cells were treated with 100 μM MDMP for 2 h then processed for immunofluorescence to detect SGs and P-bodies, as described above. The percentage of cells harboring SGs ($\geq$5 granules/cell), or P-bodies (PBs) ($\geq$4 bodies/cell) is indicated to the right of the figure and is representative of the analysis of five different fields in three independent experiments for a total of 1000 cells counted. P-bodies pictures were taken using a 60× oil immersion objective. All other pictures were produced with a 40× objective. (G) Western blot analysis of FMRP and G3BP proteins after MDMP treatment.

impairs the assembly of 80S complexes on the CrPV IRES. The TC inhibitor NSC 119893 (NSC + CHX) does not elicit this inhibitory effect because CrPV initiates translation in an eIF2α-independent manner. Moreover, it was shown that by inhibiting TC formation NSC 119893 increases the pool of free 40S ribosome available for efficient binding of CrPV (Robert *et al.*, 2006). Our results show that D-MDMP [CHX + NSC + MDMP (D)] but not L-MDMP [CHX + NSC + MDMP (L)] significantly abrogated 80S complex formation, as observed in the presence of NSC 119893 (Figure 6A), although this inhibitor effect was not complete. These results are however consistent with the previously reported action of MDMP in preventing translation initiation by inhibiting the 60S recruitment step. Targeting this step is expected to inhibit both cap-dependent and IRES-mediated translation. We tested the effect of MDMP on translation of the bicistronic mRNA Ren/CrPV/FF by using Krebs-2 extracts (Figure 6B). D-MDMP but not L-MDMP inhibits both cap-dependent translation of *Renilla* and IRES-driven translation of firefly. D-MDMP (100 μM) reduces translation of *Renilla* to 80% and translation of firefly to 50%. Similar results were obtained using other bicistronic mRNAs such as Ren/hepatitis C virus (HCV)/FF or Ren/EMCV/FF (data not shown). Thus, MDMP blocks both cap-dependent and IRES-mediated translation of mRNAs, although with different efficiency (see *Discussion*). Consistent with the role of MDMP

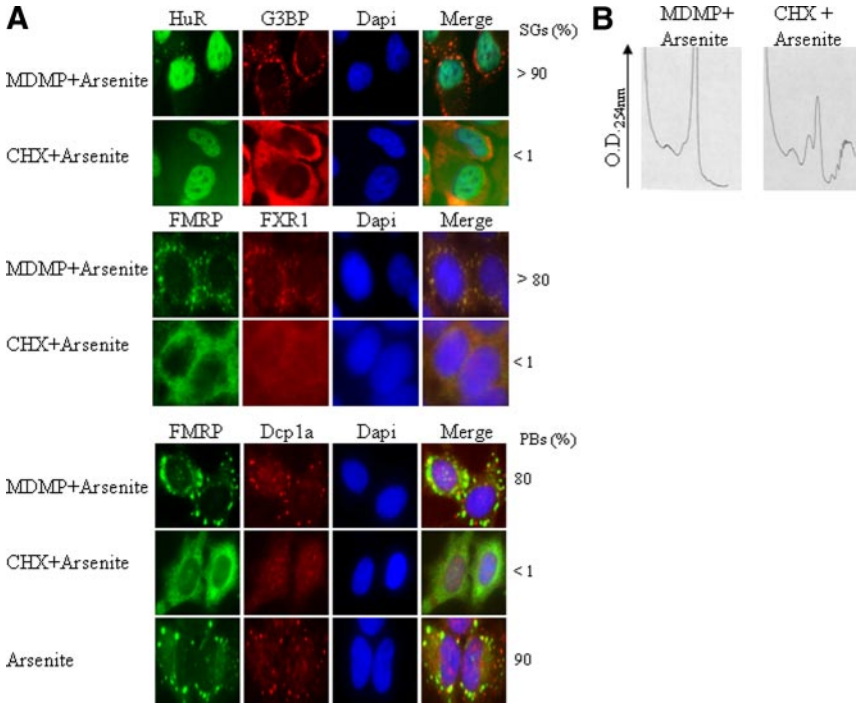

Figure 7. MDMP does not affect SG assembly in HeLa cells. (A) HeLa cells were treated with 100 μM MDMP or 50 μg/ml cycloheximide for 2 h and then 0.5 mM arsenite was added for 30 min. Cells were subjected to immunofluorescence to visualize SGs and P-bodies (A) or harvested for polysome analysis (B). The percentage of cells harboring SGs ($\geq$5 granules/cell), or P-bodies (PBs) ($\geq$4 bodies/cell) is indicated to the right of the figure and is representative of the analysis of five different fields in three independent experiments for a total of 1000 cells counted.

in targeting in vitro translation initiation downstream of 48S complex formation, no eIF2α phosphorylation was detected upon MDMP treatment (Figure 6C); yet, it strongly inhibit general translation as determined by in vivo synthesis of [^{35}S]methionine-labeled protein (Figure 6D). In contrast, L-MDMP has no effect on translation (Figure 6D). Moreover, and consistent with previous studies (Blume and Shapiro, 1989), D-MDMP but not L-MDMP, causes the disappearance of most polysomes, indicating an inhibition at the level of translation initiation (Figure 6E). We then assessed the effect of MDMP in inducing SGs. HeLa cells were incubated with MDMP under the same conditions that prevent translation (Figure 6, D–F), and the formation of SGs was assessed by immunofluorescence using various SG markers. No SGs was detected upon MDMP treatment (Figure 6F). This is not due to an altered expression of SG markers over the short course of this experiment because MDMP does not affect the level of different SG markers such as FMRP and G3BP (Figure 6G). The same results were obtained using other human transformed cell lines (data not shown). Moreover, at the conditions used in this study, MDMP treatment does not change the percentage of cells having P-bodies as assessed by localization of different P-body markers (Figure 6F, bottom right and Supplemental Figure 1).This indicates that the rate of mRNA degradation that occurs at these sites is not altered by the MDMP. We found that MDMP treatment had only a slight effect on cellular mRNA levels of individual genes as assessed by qRT-PCR (Supplemental Figure 2). Overall, our results show that targeting translation initiation at the level of the 60S joining with MDMP neither induces SGs nor affects P-bodies in HeLa cells.

Formation of SGs Can Occur in the Absence of Polysomes

Previous studies have linked the formation of SGs to polysome disassembly. It was reported that freezing polysomes using translation elongation inhibitors prevents SG assembly (Anderson and Kedersha, 2008). This suggests that the recruitment of mRNPs from polysomes is a prerequisite for the formation of SGs. To test this hypothesis, cells were first incubated with MDMP to deplete polysomes, and then arsenite was added and SG formation was assessed. In this case, SGs were efficiently induced by arsenite (Figure 7A) in absence of polysomes (Figure 7B). In contrast, and as reported previously (Kedersha *et al*., 2000), pretreatment of cells with the translation elongation inhibitor CHX blocked both SG formation and polysome disassembly upon addition of arsenite (Figure 7, A and B). Moreover, and in contrast to CHX, treatment of cells with MDMP does not affect P-bodies (Figure 7A, bottom, and Supplemental Figure 1). We concluded that SG formation can occur in the absence of polysomes.

DISCUSSION

In this study, we investigated the formation of SGs in HeLa cells under conditions that target different eIFs and different steps of translation initiation. We demonstrated that 1) depletion of TC can trigger SG formation independently of eIF2α phosphorylation; 2) reduction in the levels of eIF4B, eIF4H, or PABP leads to the induction of SGs; 3) reduction in levels of the cap-binding protein eIF4E only weakly induces SG formation; and 4) interfering with the 60S recruitment step does not trigger SG assembly or prevent SG induction. Our study demonstrates that SG formation can be uncoupled from inhibition of translation initiation.

It is well known that under stress conditions, phosphorylation of eIF2α and the subsequent TC inhibition trigger SG formation. Our results dissociate these two events in SG formation. We show that either depletion of eIF2α or disruption of its association with tRNA-Met, by using the NSC 119893 drug, is sufficient to trigger SGs (Figure 1). The percentage of cells forming SGs upon depletion of eIF2α (6–8%) is, however, lower than the percentage of those harboring SGs induced by NSC 119893. It is likely that the formation of SGs requires full depletion of eIF2α and that this can be achieved only in a small percentage of siRNA-

treated cells. Nonetheless, our results indicate that the level of TC rather than eIF2α phosphorylation status per se is determinant for SG formation. It was shown that under certain conditions, phosphorylation of eIF2α is not sufficient to trigger SG formation. We reported previously that under prolonged proteasome inhibition, SGs cannot be induced upon addition of arsenite despite a sustained high level of eIF2α phosphorylation (Mazroui *et al*., 2007). Others showed that infection with viruses such as West Nile virus do not induce SG assembly despite eIF2α phosphorylation (Emara and Brinton, 2007). More recently, it was shown that in rotavirus-infected cells, even though eIF2α is phosphorylated, SGs are not formed and that even infected cells were refractory to SG formation upon arsenite treatment (Montero *et al*., 2008). These studies suggested that some viruses prevent SG formation to allow translation of their mRNAs. It would be interesting to test whether NSC 119893 blocks replication of these viruses in infected cells by inducing SG formation.

SGs are considered as translational silencing sites of mRNA whose translation is cap dependent. Whether SG formation represses mRNAs whose translation is cap independent, such as those driven by IRESs, remains unclear. Although NSC-mediated depletion of TC prevents cap-dependent translation, it has little or no effect on translation of mRNAs driven by viral IRES such as HCV and CrPV (Robert *et al*., 2006). This suggests that formation of NSC-mediated SGs may not alter translation of such mRNAs. In contrast, translation of IRES-driven mRNAs such as EMCV (encephalomyocarditis virus) or poliovirus IRES requires the activity of both TC and eIFs (Pestova *et al*., 2001; Cencic *et al*., 2007). In these cases, SGs induced by depletion of TC or inactivation of eIFs such as eIF4F could impair translation of such IRES-containing mRNAs. Further studies are needed to clarify the effects of SGs on cap-independent translation.

We have previously shown that inactivation of eIF4A or eIF4G triggers SG assembly independently of eIF2α phosphorylation and suggests that stable 43S-mRNA association is not required for SG assembly (Mazroui *et al*., 2006). Our results herein show that depletion of eIF4B, eIF4H, or PABP, induces SG formation. The percentage of cells forming SGs upon depletion of either of these factors is however low (6–8%). It is likely that SGs become visible only in cells in which depletion of eIF-4B, eIF4H, or PABP approaches 100%. This also could explain why depletion of eIF2α (this study) or eIF4A (Mazroui *et al*., 2006) by siRNAs resulted in SG formation only in a small percentage of cells. Chemical inactivation of either eIF2α (this study) or eIF4A (Dang *et al*., 2006; Mazroui *et al*., 2006) resulted, however, in the formation of SGs in >90% of treated cells. Unfortunately, we cannot assess the formation of SGs after chemical inactivation of eIF4B, eIF4H, or PABP owing to the lack of specific compounds that target each of these factors.

Our results here show that depletion of either eIF4B or eIF4H induces SG formation, further supporting the assumption that stable ribosome–mRNA association is dispensable for SG formation. Stable ribosome–mRNA association is also thought to be mediated by mRNA circularization through the interaction of PABP and eIF4G (Craig *et al*., 1998; De Gregorio *et al*., 1999; Michel *et al*., 2000; Wakiyama *et al*., 2000). In this model, PABP-bound to the 3′ end of mRNA interacts with eIF4G bound to eIF4E positioned at the 5′ cap mRNA. Depletion of PABP is expected to disrupt the close looping of mRNA and consequently to inhibit the ribosome–mRNA association, although we have not directly shown this. The induction of SGs upon PABP depletion indicates that mRNA circularization is dispensable for SG formation.

eIF4E is responsible for cap recognition and binds with high affinity to this structure to enhance cap-dependent translation when associated with eIF4G (Sonenberg, 2008), yet its depletion does not induce SGs. Our results indicate that the two processes, the formation of SGs and the inhibition of cap-dependent translation initiation can be uncoupled. Recent studies showed that disruption of eIF4E–eIF4G interaction by the small molecule 4EGI-1 inhibits cap-dependent translation (Moerke *et al*., 2007). At high concentration, however, 4EGI-1 elicited some nonspecific effects. Our study showed that 4EGI-1 inhibits translation yet induces SG formation in only a small proportion of HeLa cells. This contrast with eIF4A inhibitors, such as hippuristanol and pateamine, and with the inhibitor of TC formation NSC 119893, which induce SG assembly in >90% of treated cells. Although we do not know at this stage if the effect of 4EGI-1 is cell cycle specific, our studies suggest that disruption of the eIF4F-5′ cap–mRNA association through inactivation of eIF4E is at best a weak inducer of SG. The possibility that eIF4E is required for SG formation remains to be investigated. eIF4E can accumulate into SGs, suggesting that it may play a role in cell stress response, for example, by recruiting capped mRNAs to SGs. Alternatively, eIF4E could promote SG formation indirectly by preventing the translation of cap-independent mRNAs whose products could block the formation of SGs. Indeed, previous studies showed that eIF4E can serve as a potent negative regulator of cap-independent translation (Svitkin *et al*., 2005). In this context, it is worthwhile to mention that in mitotic cells, where cap-dependent translation is reduced in favor of cap-independent translation (Pyronnet *et al*., 2000; Pyronnet *et al*., 2001), SGs cannot be induced (Sivan *et al*., 2007).

Our study linked the assembly of SGs to the reduced rate of TC formation and decreased levels of factors involved in the ribosome recruitment step. Both of these steps are required for the formation of the 48S preinitiation complex. Targeting the subsequent 60S joining step by the MDMP compound does not induce SG formation; yet, it inhibits both cap-dependent and IRES-mediated translation initiation, although with different efficiency. The mechanism by which the 60S associates with the 40S on an IRES is still a matter of debate. Clearly, there is no evidence that the subunit joining that occurs on an IRES is identical to the joining that occurs in a cap-dependent process. Hence, we cannot expect the compound to inhibit the process to the same extent in both initiation pathways. Another possibility that might explain the difference in the translation of the cap and CrPV-driven reporters could be due to the fact that by binding the 40S, the IRES might induce a conformational change in the ribosomal subunit that would alter the binding of MDMP. Alternatively, differences in initiation rates through the cap and the IRES could explain the differences in sensitivity to MDMP. If the IRES has the ability to drive more initiation than the cap, or to allow 60S joining more efficiently, and then a higher concentration of compound should be required to inhibit the IRES to the same extent. Clearly, identification of translation initiation factors that are targeted by MDMP should provide further insight into the mechanisms by which this component inhibits the 60S joining step in translation initiation.

Recently, a novel pathway of SG formation was identified (Ohn *et al*., 2008). It was shown that the acetylglucosamination (O-GlcNAc) modification of ribosomal proteins that is induced by arsenite promotes the formation of SGs. However, it is not clear whether upon stress, O-GlcNAc modification promotes the initial translation repression that is mediated by stress or the subsequent assembly of SGs.

Investigating this modification under conditions that inhibit translation initiation without inducing SGs, for example, under MDMP treatment, should clarify the mechanisms by which O-GlcNAc promotes SG formation.

It was shown that blocking polysome disassembly by translation elongation inhibitors prevents the formation of SGs (Anderson and Kedersha, 2008). This led to the hypothesis that polysome disassembly might be required for the formation of SGs. Our data show that SGs can be efficiently induced in cells in which polysomes were depleted by pretreatment with MDMP, indicating that the formation of SGs is not a consequence of polysomes disassembly. The formation of SGs in the absence of polysomes is intriguing but may reflect an antagonistic role of polysome components, such as the 60S ribosomal subunits in SG formation.

The formation of SGs was recently shown to suppress cancer cell death in response to genotoxic drugs that could explain the previously described role of SGs in promoting tumor resistance to radiation therapy (Moeller *et al*., 2004; Arimoto *et al*., 2008). In this context, further dissection of the mechanism of SG assembly is important to better design anticancer strategies, for instance that can target translation initiation without inducing these "prosurvival" SGs. Our study identifies windows in translation initiation that can be targeted without SG induction.

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